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Formation of Dityrosine Cross-Links during Breadmaking

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To establish its significance during commercial breadmaking, dityrosine formation was quantified in flours and doughs of six commercial wheat types at various stages of the Chorleywood Bread Process. Dityrosine was formed mainly during mixing and baking, at the levels of nmol/g dry weight. Good breadmaking flours tended to exhibit a higher dityrosine content in the final bread than low quality ones, but no relationship was found for dityrosine as a proportion of flour protein content, indicating that the latter was still a dominant factor in the analysis. There was no correlation between gluten yield of the six wheat types and their typical dityrosine concentrations, suggesting that dityrosine cross-links were not a determinant factor for gluten formation. Ascorbic acid was found to inhibit dityrosine formation during mixing and proving, and it has no significant effect on dityrosine in the final bread. Hydrogen peroxide promoted dityrosine formation, which suggests that a radical mechanism involving endogenous peroxidases might be responsible for dityrosine formation during breadmaking.

KEYWORDS: Wheat; breadmaking; dough; flour; tyrosine; dityrosine; cross-link; HPLC; ascorbic acid

INTRODUCTION

Dityrosine is an oxidatively coupled dimer of tyrosine and comprises two tyrosine units linked via a C−C biphenyl bond. It possesses a characteristic intense fluorescence, with an emission maximum near 400 nm. Gross and Sizer (*1*) described for the first time the in vitro formation of dityrosine by oxidation of tyrosine in the presence of horseradish peroxidase and hydrogen peroxide. Andersen (*2*) then reported the presence of tyrosine cross-links in proteins in vivo after isolating dityrosine from resilin, an elastic protein similar to elastin that is present in insect cuticles. Subsequently, dityrosine cross-links have been shown to occur in several structural animal proteins, including elastin (*3*), fibroin (*4*), keratin (*4*), cuticlin (*5*), and collagen (*6*). In each case, dityrosine has been found to be partially responsible for the elastic and insoluble properties of the respective protein. In plants, cross-links between tyrosine residues are known to form part of the structure of plant cell walls and they can be found as isodityrosine, an isomer of dityrosine, with the two tyrosine residues linked together by a diphenyl ether bond (*7*).

Apart from the enzymatic oxidation of tyrosine, dityrosine formation has been found as a result of UV (*8*) and γ-radiation (*9*), exposure to oxygen free radicals (*10*, *11*), or other posttranslational processes affecting specific structural proteins (*3*, *12*). Dityrosine can be used as a marker for oxidative stress and protein damage (*13*, *14*), aging (*15*, *16*), and disease (*17*, *18*).

The formation of dityrosine cross-links in wheat proteins, specifically gliadins, was first demonstrated in vitro by Aeschbach et al. (*19*). Later on, the first evidence for the native formation of dityrosine cross-links in wheat gluten proteins was provided (*20*). This research proposed that dityrosine bonds were technologically important in the breadmaking process and that they contribute to gluten structure formation during the mixing of dough. More recently, dityrosine has been quantified in wheat flour and dough, and the influence of some bread improvers has been investigated (*21*). This latter study concluded that dityrosine bonds are likely to play a minor role in the process of dough formation.

In this study, we have quantified dityrosine at various stages (mixing, molding, proving, and baking) of breadmaking using the Chorleywood Bread Process (CBP) in order to establish the processing factors that influence dityrosine formation. Canadian Western Red Spring (CWRS) and five commercial U.K. wheat varieties of varying breadmaking quality have been studied to seek correlations between dityrosine levels and varietal breadmaking quality. The techniques most commonly used for the analysis of dityrosine are reversed phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection (*13*, *14*, *20*, *22*), gas chromatography coupled with mass spectrometry (*23*, *24*), and liquid chromatography coupled with mass spectrometry (*21*, *25*). A simplified method for the analysis of

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dityrosine in dough systems based on the former method of RP-HPLC with fluorescence detection is reported.

MATERIALS AND METHODS

Chemicals. L-Tyrosine and horseradish peroxidase were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom); acetonitrile (HPLC super gradient grade) was from Labscan Ltd. (Stillorgan, Co. Dublin, Ireland). All other chemicals were obtained from either Fisher Scientific UK Ltd. (Loughborough, Leicestershire, United Kingdom) or Sigma Chemical Co. and were of the highest purity available.

Synthesis of Dityrosine. Dityrosine was synthesized by the horseradish peroxidase-catalyzed oxidation of L-tyrosine according to the method described by Malencik et al. (*26*). The identity and purity of the product obtained were confirmed by ^{1}H and ^{13}C NMR spectroscopy and were performed with a Bruker AMX 400 spectrometer, operating at a frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C analysis, using D_2O as the solvent.

Wheat Flour. Six wheat flour samples were used emanating from the following wheat types: CWRS and the commercial U.K. varieties Hereward, Rialto, Soissons, Savannah, and Claire. A range of properties for each of the flours were analyzed as well as their performance in a standard test baking method based on the CBP. CWRS, Hereward, Soissons, and Rialto are all breadmaking wheat types with CWRS having the highest protein content and dough strength. Hereward typically gives weaker dough properties than CWRS although it performs very well under CBP conditions. Soissons was selected as it can exhibit high levels of gluten strength while Rialto is an example of a breadmaking variety having the 1B 1R translocation. Claire and Savannah are nonbreadmaking varieties with Claire being soft milling and typically used for the production of biscuits and cakes while Savannah is a hard milling feed wheat.

Characterization of Flour Properties. All flours were commercially milled in accordance with the requirements for breadmaking flours in the United Kingdom. Flour properties were assessed in terms of protein content, quality, and dough rheological properties. The flour protein content was determined by the Dumas method in accordance with CCFRA (*27*), while the remaining analyses were performed as detailed in Alava et al. (*28*).

Small-Scale Mixing Procedure. A 10 g Mixograph (National Manufacturing Co., Lincoln, NE) was used at the optimum water absorption of each flour as determined by Farinograph (*28*). Mixing was carried out for 1, 5, and 10 min. The dough was then frozen and lyophilized prior to RP-HPLC analysis.

Bread Production. A method based on the CBP was used for baking assessment. The base formulation used in all cases in baker's percentages was as follows: 100% flour, water to Farinograph water absorption (600 line), 2.5% yeast, 2% salt, 1% bread fat, and fungal α-amylase to give an activity of 0.80 Ceralpha units per gram of flour. Doughs were produced both with and without ascorbic acid at 100 ppm (on a flour basis) and were mixed in a Morton Z-blade mixer to a final work input of 11 W h/kg. Final dough temperatures were controlled to 30.5 ± 1 °C. Doughs were then divided into 930 g units, molded into a ball, and subjected to a 7 min intermediate proof. Subsequently, doughs were molded to give the final shape (four-pieced), placed in bread tins, and proved to height at 43 °C. The doughs were then baked in an oven at 244 °C for 25 min. Samples were taken after mixing, intermediate proof, final molding, final proof, and baking, then frozen, and lyophilized prior to further analysis.

Gluten Isolation. An automatic gluten washing apparatus (Glutomatic 2100, Perten Instruments AB, Huddinge, Sweden) was used for the isolation of gluten from 10 g of flour at the optimum water absorption according to Farinograph. The flour was mixed in the apparatus for 30 s prior to the washing step. Gluten samples were then frozen and lyophilized. Additional ingredients were added directly to the flour or dissolved in water with the exception of BHA (3-*tert*-butyl-4-hydroxyanisole), which was dissolved in petroleum ether.

HPLC Analysis. Hydrolysis of the flour, dough, and bread samples was performed in 6 N HCl with 1% phenol for 24 h at 110 °C. The lyophilized dough and bread samples were ground with a Perten KT 3100 Falling Number Mill (Perten Instruments AB) with a 0.8 mm screen. Twenty milligrams of the lyophilized material was weighed and placed in a tube (Fisher glass round tubes 13 × 100 with screw caps resistant to heat), and 2 mL of 6 N HCl with 1% phenol was added. Tubes were then flushed with nitrogen and placed in a heating block at 110 °C for 24 h. Afterward, tubes were placed in a Genevac EZ-2 and EZ-2^{plus} evaporation system until total evaporation of the HCl and phenol was achieved. Samples were then reconstituted in 0.4 mL of HPLC water containing 0.1% trifluoroacetic acid (TFA), filtered, and loaded into the HPLC system. Amino acid analysis of the hydrolyzed dough and flour was carried out using a RP-HPLC method adapted from Tilley et al. (*20*). A stepwise gradient with acetonitrile and water containing 0.1% TFA was used (3, 10, 40, 95, and 95 and 3% acetonitrile at 0, 35, 50, 60, 65, and 85 min, respectively) on a C-18 column Ace 5 AQ (25 cm × 4.6 mm, Hichrom, Theale, Berkshire, United Kingdom). The column temperature was 30 °C, and the flow rate was 0.7 mL/min. A fluorescence detector at $\lambda_{ex} = 285$ nm and $\lambda_{em} = 405$ nm and a diode array detector at 285 nm were used.

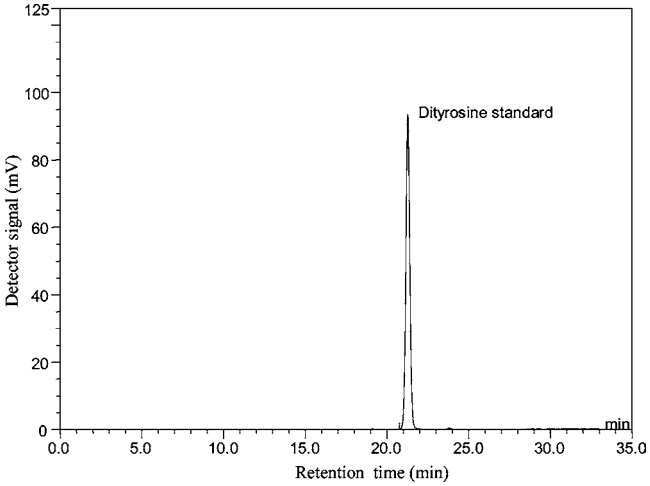

Figure 1. RP-HPLC chromatogram showing the elution time for the dityrosine standard with fluorescence detection at $\lambda_{ex} = 285$ nm and $\lambda_{em} = 405$ nm.

Data Analysis. Statistical analysis was performed using SPSS and SAS software. Univariate analysis of variance and orthogonal contrast were used to compare the significant difference among means. Samples were analyzed in triplicate in all of the cases except for the breadmaking study, for which five replicates were performed.

RESULTS AND DISCUSSION

Synthesis of Dityrosine. The purity of the synthesized dityrosine was verified by NMR spectroscopy with the following signals. ^{1}H NMR: δ 2.95 (2H, dd, $J = 7.29, 14.72$), 3.05 (2H, dd, $J = 5.42, 14.72$), 3.95 (2H, dd, $J = 5.43, 7.21$), 6.78 (2H, d, $J = 8.3$), 6.91 (2H, d, $J = 2.28$) and 7.00 (2H, dd, $J = 2.33, 8.32$). ^{13}C NMR: δ 37.7, 57.7, 119.0, 128.4, 129.1, 133.2, 135.0, 155.3, and 175.3. These results agree with data reported by Ushijima et al. (*29*), Briza et al. (*30*), and Hanft and Koehler (*21*). A single peak was obtained after the product was loaded onto the HPLC system optimized for the analysis of dough and bread samples (**Figure 1**).

Validation of the Method. The recovery of the method was calculated by adding different amounts of dityrosine standard to the samples before hydrolysis and was found to be 99.0% with a standard deviation of 5.1%. The limit of detection and limit of quantitation were calculated according to Miller and Miller (*31*). The limit of detection for dityrosine was 0.10 nmol/g sample, and the limit of quantitation was 0.22 nmol/g sample. Both of these were below the range of the samples analyzed in this study. Standard calibration was performed using seven concentrations between 10 and 600 ng/mL.

Table 1. Quality Characteristics and Test Baking Performance of the Flour Samples Evaluated

	wheat variety or class					
	Hereward	CWRS	Rialto	Soissons	Savannah	Claire
protein content (%) dmb[a]	12.4	16.0	12.3	11.1	9.6	9.8
Hagberg falling number (s)	319	371	280	266	288	223
water absorption (%) as is[b]	58.8	60.7	58.7	54.6	56	51.4
development time (min)[b]	2.5	5	3.5	2	2.5	1.5
stability (min)[b]	4	8	4	3	4.5	2.5
degree of softening (BU)[b]	110	60	100	120	80	150
gel protein mass per 5 g flour (g)	9.3	11.7	5.4	7	3.5	6.3
gel protein elastic modulus (Pa)	26.5	50.4	16.3	20.5	29.6	34.6
CBP loaf volume (ml)[c,d]	3284	3428	3085	3166	2173	2879
gluten formed (mg/g flour)[e]	54.7	97.6	58.9	50.8	6.9	46.0

[a] $N \times 5.7$. [b] Results produced by Brabender Farinograph operated to the 600 line. [c] CBP loaf volume normalized to a standard 800 g loaf weight. [d] Doughs produced using ascorbic acid at 100 ppm on flour basis. [e] Gluten produced using a Glutomatic 2100 apparatus, expressed in mg of gluten per gram of flour.

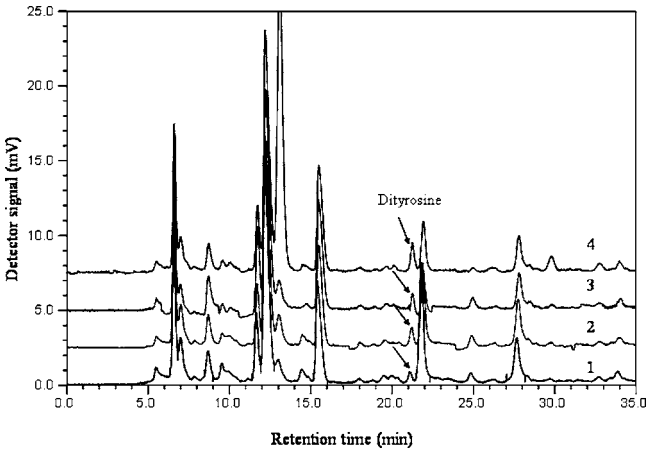

Figure 2. RP-HPLC chromatograms with fluorescence detection obtained from the analysis of Hereward flour and dough mixed for 1, 5, and 10 min (chromatograms number 1, 2, 3, and 4, respectively). The dityrosine peak eluted at 21 min.

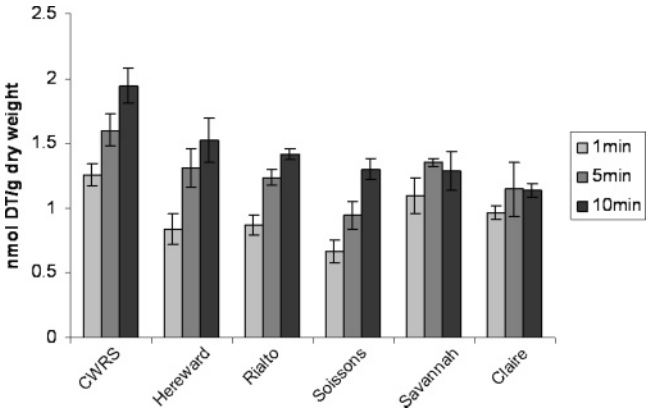

Figure 3. Dityrosine concentration in flour and dough mixed for 1, 5, and 10 min in a 10 g mixograph, expressed in nmol/g dry weight. Results are shown for the wheat flour types CWRS, Hereward, Rialto, Soissons, Savannah, and Claire.

Flour Quality Parameters. As would be anticipated given the difference in intended end uses, there were significant differences in properties between the flours (**Table 1**). Those destined for breadmaking (Hereward, CWRS, Rialto, and Soissons) exhibited higher protein contents and greater loaf volumes than the nonbreadmaking varieties. Equally, the hard milling wheats (Hereward, CWRS, Rialto, Soissons, and Savannah) gave higher levels of water absorption as assessed by Farinograph and as would be expected. It was surprising to note, however, that the breadmaking performance obtained overall was poor, with lower than anticipated loaf volumes for Hereward, CWRS, Rialto, and Soissons. In addition, the gluten characteristics of Soissons as assessed by evaluating the gel protein fraction were poorer than those expected. Higher quantities of gel protein allied to larger elastic moduli are believed to be indicative of greater protein strength with elastic moduli in the range of 15−40 Pa generally giving good breadmaking performance for CBP (*28*). The gel protein results for Claire were higher than anticipated while the dough properties of Savannah were stronger than expected for feed wheat. Overall, the test baking results ranked the flour samples in a manner consistent with previous experience of these flour types, although in some cases the flour and dough properties were not those expected for the variety in question.

Dityrosine during Dough Mixing. Dityrosine was identified in all of the chromatograms as the peak eluting at approximately 21 min (**Figure 1**). **Figure 2** shows the chromatograms obtained for Hereward flour, and doughs produced from Hereward flour after 1, 5, and 10 min of mixing. Dityrosine levels increased during mixing from 0.935 to 1.704 nmol/g dry weight. The peak at 21 min also increased as the mixing time increased for the other five wheat types studied (**Figure 3**). The flours giving the best breadmaking performance (CWRS and Hereward) exhibited the greatest dityrosine increase during mixing, followed by Rialto and Soissons. Claire flour had the lowest dityrosine concentration, and this did not increase significantly as the mixing time increased. Savannah, which is a low quality wheat variety, also did not exhibit a big increase in dityrosine bonds during mixing, although it had a dityrosine content similar to that for the good breadmaking flours. Therefore, on the basis of these results, wheat quality and dityrosine concentration during mixing do not appear to correlate.

Dityrosine Formation during the CBP. The results for the breadmaking study are represented in **Figure 4**. The initial dityrosine content of flours (**Figure 4a**) ranged between 0.441 and 0.773 nmol/g dry weight, with Hereward and CWRS having the lowest dityrosine content, followed by Claire, Soissons, Rialto, and Savannah. During mixing, in agreement with the previous study, the dityrosine levels increased, with concentrations ranging between 0.937 and 1.122 nmol/g. During proving and molding, there were no significant variations in dityrosine levels, with concentrations after final proof being between 0.950 and 1.342 nmol/g dry weight, which were only slightly higher than after the mixing stage. After baking, levels of dityrosine in bread were increased with concentrations ranging from 1.623 to 2.280 nmol/g dry weight. Statistical analysis showed that dityrosine content in bread was higher for CWRS and Hereward,

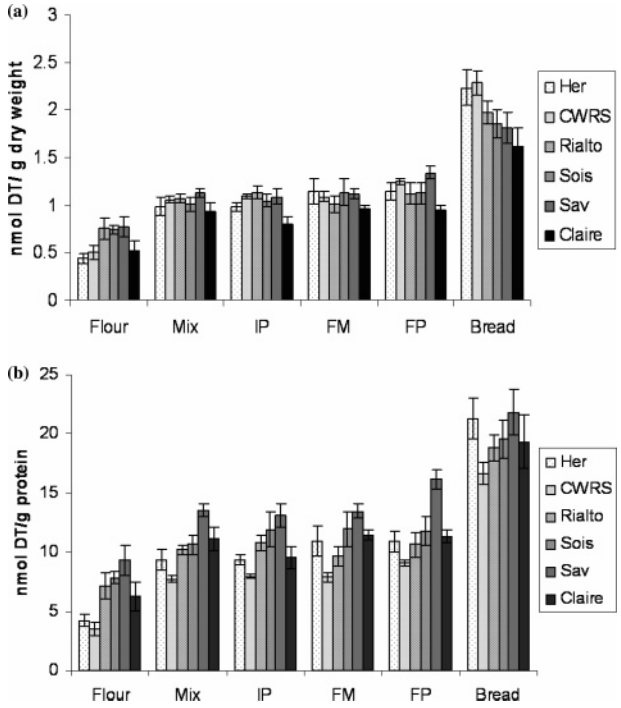

Figure 4. Dityrosine concentration expressed as (**a**) nmol/g dry weight and (**b**) nmol/g protein in flour and during the different stages of the CBP [mixing (Mix), intermediate proof (IP), final mold (FM), final proof (FP), and bread]. Results are shown for the wheat flour types CWRS, Hereward (Her), Rialto, Soissons (Sois), Savannah (Sav), and Claire.

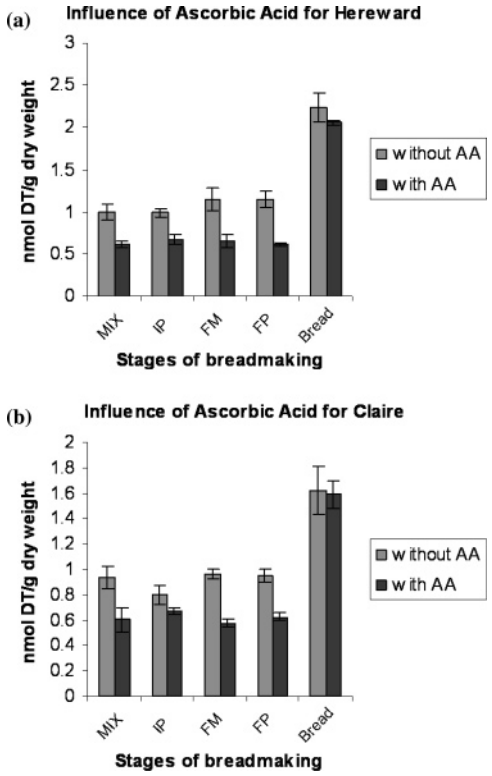

Figure 5. Dityrosine concentration in (**a**) Hereward flour and (**b**) Claire flour during the different stages of the CBP [mixing (MIX), intermediate proof (IP), final mold (FM), final proof (FP), and bread] with and without the addition of ascorbic acid. Results are expressed in nmol/g dry weight.

followed by Rialto and Soissons, with Claire and Savannah having the lowest concentrations. Dityrosine levels, therefore, appeared to correlate to some extent with the breadmaking potential of the flours. However, the differences were small, and it is important to note that the low quality Savannah variety had similar or even higher concentrations than the good breadmaking flours in the other stages of the CBP including mixing.

The levels of dityrosine obtained in this study were in excellent agreement with the levels obtained in flour and dough by Hanft and Koehler (*21*) when expressed as a proportion of the mass of material assessed. In addition to reporting results in this way, however, the levels of dityrosine obtained were also standardized with respect to protein content (**Figure 4b**). A different trend was observed, with Savannah having the highest dityrosine content per gram of protein during the different stages of breadmaking, followed by Rialto and Soissons, and with CWRS having the lowest concentration. These results do not correlate dityrosine levels with breadmaking potential in wheat varieties. Therefore, it seems that the dominant factor is protein content rather than the level of dityrosine cross-linking.

The addition of ascorbic acid had no significant effect on dityrosine levels in bread, but in the earlier stages of breadmaking, it appeared to inhibit the formation of dityrosine (**Figure 5**). Therefore, ascorbic acid does not appear to act by oxidizing tyrosine to dityrosine, which contradicts observations reported by Tilley et al. (*20*). According to these authors, an increase in the dityrosine peak was observed after mixing and heating when ascorbic acid was added to the flours. However, the results reported agree with those of Hanft and Koehler (*21*), who also observed a slight inhibitory effect of ascorbic acid in dityrosine formation.

Influence of Dityrosine on Gluten Formation. The amount of gluten obtained with the Glutomatic apparatus was consistent

Table 2. Dityrosine Concentration in Gluten

wheat type	dityrosine concn (nmol/g dry gluten)
CWRS	8.27 ± 0.30
Hereward	8.99 ± 1.04
Rialto	9.57 ± 0.61
Soissons	7.49 ± 0.91
Savannah	10.21 ± 0.26
Claire	6.03 ± 0.64

with the protein content of each wheat type (**Table 1**), with CWRS having the highest amount of gluten formed (97.6 mg/g flour) and Savannah the lowest (6.9 mg/g flour). Hereward, Rialto, Soissons, and Claire formed similar amounts of gluten (between 46.0 and 58.9 mg/g flour). Interestingly, the amount of gluten formed showed no correlation to the amount of dityrosine formed per gram of gluten. Indeed, the amount of gluten formed varies dramatically (14-fold) between the lowest and the highest values obtained whereas the corresponding dityrosine concentrations (**Table 2**) showed relatively little variation (less than 2-fold). These data suggest that dityrosine cross-links are not a determinant factor in the structure of gluten.

Influence of Additives on Dityrosine Formation. Following the observation that ascorbic acid showed a mild inhibitory effect on the formation of dityrosine, the effects of the addition of other materials were assessed. The addition of radical scavengers (cysteine, glutathione, and BHA) to gluten produced a small decrease in dityrosine concentration (**Table 3**). Adding tyrosine and horseradish peroxidase to the flour when producing gluten also led to a decrease in dityrosine content. However, when hydrogen peroxide was added, a large increase in dityrosine concentration was observed. It is well-documented that when peroxidase and hydrogen peroxide react with tyrosine, dityrosine

Table 3. Dityrosine Concentration in Gluten as Affected by Additives

CWRS gluten with additives	dityrosine concn (nmol/g dry gluten)
gluten	8.27 ± 0.30
gluten with ascorbic acid (100 ppm)	5.81 ± 0.45
gluten with potassium bromate (100 ppm)	4.95 ± 0.44
gluten with cysteine (100 ppm)	5.03 ± 0.94
gluten with glutathione (100 ppm)	5.85 ± 1.35
gluten with BHA (100 ppm)	5.51 ± 2.18
gluten with hydrogen peroxide (100 ppm)	7.97 ± 0.97
gluten with hydrogen peroxide (6022 ppm)	27.79 ± 0.84
gluten with horseradish peroxidase (0.71 mg)	6.52 ± 0.57
gluten with tyrosine 0.1% (w/w)	6.04 ± 0.86

is the main product formed (*1*, *19*). In the presence of hydrogen peroxide, tyrosine probably reacts with the endogenous peroxidase of wheat forming dityrosine. During baking, peroxidase could still form dityrosine as peroxidases are unusually heat stable enzymes (*32*). The mechanism proposed for dityrosine formation is a phenolic coupling of two phenoxy radicals of tyrosine, which also explains the effect of radical scavengers on dityrosine. However, the presence of hydrogen peroxide in dough has never been proved (*33*), although it has been suggested that hydrogen peroxide can be formed during fermentation in the presence of yeast (*34*). This would explain the increase of dityrosine concentration during baking but would be unlikely to explain the formation of dityrosine during mixing in the CBP at which stage yeast fermentative activity would be minimal. Fry (*35*) has recently questioned whether peroxidase action on phenolic compounds occurs in vivo and has suggested other enzymic and nonenzymic oxidative systems as an alternative. Therefore, it is possible that dityrosine is formed by the action of other enzymes present in wheat or via other mechanisms.

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